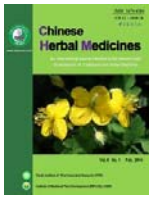

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Letter

# Isolation and Identification of Bioactive Constituents from Stem Barks of *Illicium difengpi*

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ABSTRACT

**Objective** To isolate and identify bioactive constituents from the stem barks of *Illicium difengpi*. **Methods** The chemical constituents were isolated and purified by repeated silica gel, Sephadex LH-20, recrystallization, and preparative HPLC techniques. The structures of the compounds were identified on the basis of spectral data including NMR, MS, and IR. **Results** Two sesquiterpene lactones, majucin (**1**) and anisatin (**2**), two steroids, β-sitosterol (**3**) and daucosterol (**4**), three carboxylic acids, 2-ethyldecanoic acid (**5**), shikimic acid (**6**), and 3,4-dihydrobenzoic acid (**7**), and a flavonoid, quercetin (**8**), were successively isolated from the stem barks of *I. difengpi*. **Conclusion** Apart from compound **3**, other seven compounds are reported in this plant for the first time. The results suggested that the current studies on *I. difengpi* is still far from being well known and therefore more studies need to be done for better understanding of this plant.

*Key words*

carboxylic acid; flavonoid; Illiciaceae; *Illicium difengpi*; sesquiterpene lactone



## 1. Introduction

*Illicium* L., a member of Illiciaceae family, containing about 40 species of evergreen trees and shrubs, mostly distribute in eastern and southeastern Asia and a few in southeastern North America and tropical America. Twenty-seven of these species are found in China, about 14 of them are endemic to various provinces (Yuwu, 1996). Previous chemical investigations on this genus have yielded prenylated $C_6$-$C_3$ compounds, neolignans, and a large number of unique sesquiterpene lactones exhibiting neurotoxic and neurotrophic activities (Fukuyama et al, 1993; Yokoyama et al, 2002). From a chemotaxonomic point of view, the species of *Illicium* L. are rich in biosynthetically unique *seco*-prezizaane-type

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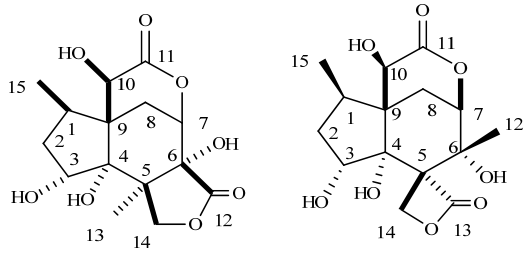
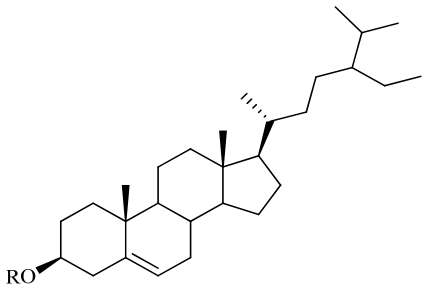
sesquiterpenes which are considered to be characteristic chemical markers (Tang et al, 2009). In addition, the prenylated $C_6$-$C_3$ compounds, referred to as phytoquinoids, are also considered to be characteristic constituents in this genus (Wu et al, 2009). *Illicium difengpi* B. N. Chang, indigenous to China, is a shrub that grows in the mountainous area of Guangxi Zhuang Autonomous Region. Its stem bark is listed in *Chinese Pharmacopeia 2010* and has been applied as a Chinese materia medica to treat rheumatic arthritis by relieving lumbago and pain in the knees (Pharmacopeia Committee of P. R. China). The plant is collected in spring and autumn, and dried in the sun or at a low temperature. Previous phytochemical studies on this plant have reported the isolation of 30 compounds including triterpene acids, phenylpropanoids, neolignans, and their glycosides (Fang, 2010; Huang et al, 1997; Kouno et al, 1993; Kouno et al, 1992). Thus, with only a few compounds having been isolated from this plant, more studies on the chemical constituents are necessary in order to establish a safety and quality control system for medicinal preparations containing this plant. Herein, we describe the component isolation and structure elucidation of two sesquiterpene lactones, majucin (**1**) and anisatin (**2**), two steroids, β-sitosterol (**3**) and daucosterol (**4**), three carboxylic acids, 2-ethyldecanoic acid (**5**), shikimic acid (**6**), and 3,4-dihydrobenzoic acid (**7**), and a flavonoid, quercetin (**8**), from the stem barks of *I. difengpi.* (Figure 1)



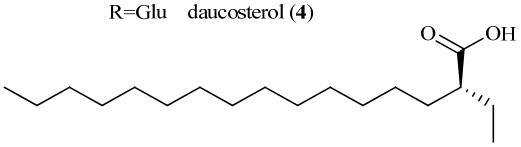
majucin (**1**)



anisatin (**2**)



R=H b-sitosterol (**3**)
R=Glu daucosterol (**4**)



2-ethylhexadecanoic acid (**5**)

**Figure 1 Structures of compounds 1—5**

## 2. Materials and methods

### *2.1 General*

Fourier transform spectrophotometer was used to detect IR spectra. The $^1$H-NMR and $^{13}$C-NMR spectra were recorded on a Bruker AV−500 Spectrometer with TMS as internal standard. The MS spectrum was performed on a VS-ZAB-HS Spectrometer. TLC was performed on silica gel $GF_{254}$ plates (Qingdao Marine Chemical Co., Ltd., China). For column chromatography, silica gel (100−200 mesh, Qingdao Marine Chemical Co., Ltd., China) and Sephadex LH-20 were used. Agilent HPLC was used to analyze and purify the samples with hypersil ODS column (5 μm). Salkowski method was used to determine the presence of steroids.

### *2.2 Plant material*

The stem barks of *Illicium difengpi* B. N. Chang (10 kg) were purchased from Guangxi Zhuang Autonomous Region in China in January 2011 and identified by Prof. Jian Zhang (College of Chinese Materia Medica, Tianjin University of Traditional Chinese Medicine). A voucher specimen (EO2011) is deposited at College of Traditional Chinese Medicine, Tianjin University of Traditional Chinese Medicine.

### *2.3 Extraction and isolation*

The air dried stem barks of *I. difengpi* (8 kg) were ground and extracted with 95% and 70% EtOH for three times (2 h for each time) under reflux. The extracts were concentrated under reduced pressure and combined. The concentrated extract was suspended in water, reduced to an appropriate level that could be handled with a separating funnel and then partitioned with petroleum ether, chloroform ($CHCl_3$), ethylacetate (EtOAc), and *n*-butanol (*n*-BuOH) successively.

The EtOAc fraction (90 g) was subjected to silica gel column (80 cm × 8 cm, 100–200 mesh) eluted with $CH_2Cl_2$-EtOAc-MeOH (100:1:0.1→1:1:0.1) gradient system to yield 10 fractions (Frs. $A_1$–$A_{10}$) on the basis of TLC analysis. Fr. $A_1$ was recrystallized from MeOH to give compound **3**. Fr. $A_2$ (3 g) was further subjected to silica gel (50 cm × 1 cm, 100–200 mesh) eluted with petroleum ether-ethyl acetate (10:1→1:1) gradient system to give five subfractions (SFrs. $A_2B_1$→$A_2B_5$). SFr. $A_2B_4$ was repacked on silica gel column and eluted with petroleum ether-ethyl acetate (1:3) isocratic solvent system to yield compound **5**. Fr. $A_4$ was purified by preparative HPLC using 6% $CH_3CN$-$H_2O$ to afford compound **7**. Fr. $A_5$ was recrystallized from methanol to give compounds **1** and **2**. Fr. $A_6$ (3 g) was subjected to silica gel column (50 cm × 2 cm, 100–200 mesh), eluted with $CH_2Cl_2$-MeOH (30:1→1:1) gradient system to afford four subfractions (SFrs. $A_6B_1$→$A_6B_4$). SFr. $A_6B_2$ was then purified by Sephadex LH-20 and eluted with MeOH-$CHCl_3$ (1:1) to yield compound **8**. SFr. $A_8$ was recrystallized from methanol to give compound **4**. Finally, Fr.

$A_9$ was purified by preparative HPLC using 3% $CH_3CN$-$H_2O$ to yield compound **6**.

## 3. Results

Compound **1** was obtained as white crystal. Its mass spectrum showed a molecular ion peak at *m/z* 328 corresponding to the molecular formula ($C_{15}H_{20}O_8$) and molecular weight. In the IR spectrum, it showed absorption due to hydroxyl groups at 3749, 3559, 3529, 3405, and 3095 $cm^{-1}$, a γ-lactone at 1772 $cm^{-1}$ and a δ-lactone at 1739 $cm^{-1}$. The $^{13}$C-NMR spectrum exhibited 15 signals, including two characteristic lactone C=O resonances at $\delta$ 176.72 and 173.58. The features of its $^{13}$C-NMR spectrum along with $^1$H-NMR spectrum were very similar to those of majucin (Table 1) (Kouno et al, 1990). Compound **1** was therefore identified as majucin.

**Table 1 $^{13}$C-NMR and $^1$H-NMR data for compounds 1 and 2 (in DMSO-$d_6$)**

| Position | Compound **1** | | Compound **2** | |
|---|---|---|---|---|
| | $\delta_C$ | $\delta_H$ | $\delta_C$ | $\delta_H$ |
| 1 | 36.7 | 2.44 | 36.7 | 2.44 |
| 2 | 41.2 | 1.73, 1.56 | 41.2 | 1.73, 1.56 |
| 3 | 71.3 | 4.29 | 71.3 | 4.29 |
| 4 | 81.0 | − | 81.0 | − |
| 5 | 50.2 | − | 50.2 | − |
| 6 | 78.3 | − | 78.3 | − |
| 7 | 78.7 | 4.96 | 78.7 | 4.96 |
| 8 | 25.4 | 1.90, 2.27 | 25.4 | 1.90, 2.27 |
| 9 | 46.0 | − | 46.0 | − |
| 10 | 69.0 | 4.97 (H), 6.48 (OH) | 69.0 | 4.97 (H), 6.48 (OH) |
| 11 | 173.6 | − | 176.7 | − |
| 12 | 176.7 | − | 19.6 | 1.23 |
| 13 | 19.6 | 1.23 | 173.6 | |
| 14 | 71.1 | 4.11, 3.92 | 71.1 | 4.12, 4.11 |
| 15 | 13.6 | 0.87 | 13.6 | 0.87 |

Compound **2** was obtained as clear crystal. Its mass spectrum showed a molecular ion peak at *m/z* 328 corresponding to the molecular formula ($C_{15}H_{20}O_8$). In the IR spectrum, absorptions due to a hydroxyl group at 3749, 3405, and 3094 $cm^{-1}$, a β-lactone at 1842 $cm^{-1}$ and a δ-lactone at 1647 $cm^{-1}$ were demonstrated. The presence of a β-lactone moiety was also indicated by the characteristic small coupling constant ($J$ = 5.5 Hz) of AB type signals at $\delta_H$ 4.12 and 4.11 in the $^1$H-NMR spectrum. The $^{13}$C-NMR spectrum also exhibited 15 signals, including two characteristic lactone C=O resonances at $\delta$ 176.72 and 173.58 as compound **1** (Table 1) (Kouno et al, 1991). It is important to note that although the NMR features of compounds **1** and **2** were very similar, their crystals were different in color and their IR spectral data were also different, especially on the C=O stretching frequencies. Whereas compound **1** is thought to have γ- and δ-lactone groups, and compound **2** has β- and δ-lactone groups. Compound **2** was therefore identified as anisatin.

Compound **3** was obtained as white crystal. The compound gave a positive test for steroid and was therefore assumed to be a compound containing a steroidal nucleus. The NMR spectra of this compound resembled that of β-sitosterol published in previous studies (Chaturvedula and Praksah, 2012; Koizumi et al, 1979; Patra et al, 2010).

Compound **4** was obtained as white powder. The compound gave positive reaction for steroid and was therefore assumed to contain a steroidal nucleus. Its NMR data were found to be identical to that of compound **3**, except for a set of sugar signals in the $^{13}$C-NMR data. The sugar was identified as *D*-glucopyranose by closely studying its signals in the $^{13}$C-NMR spectrum. Compound **4** was eventually identified as β-sitosterol-3-*O*-β-*D*-glucopyranoside (daucosterol) through comparison of its NMR data with that of daucosterol reported in the literatures (Yoo et al, 2006).

Compound **5** was obtained as clear oil. Its mass spectrum showed a molecular ion peak at *m/z* 284 corresponding to the molecular formula ($C_{18}H_{36}O_2$). Its IR absorption spectrum showed a very broad band at 3015–2676 $cm^{-1}$ due to O-H stretching. The spectrum also showed absorption peaks at 1703 $cm^{-1}$ (C=O vibration) and 1463–1022 $cm^{-1}$ regions (C-O stretching and O-H in plane bands). $^1$H-NMR ($CDCl_3$, 500 MHz) spectrum showed the absorption due to a tertiary proton at $\delta$ 3.65, methylene protons due to $-CH_2$ groups ranging from $\delta$ 1.54−2.58 and the methyl groups absorbing at a range of $\delta$ 0.797−0.822. $^{13}$C-NMR spectrum revealed the presence of a carboxylic acid carbonyl carbon absorbing at $\delta$ 177.62 (C-1) and a tertiary carbon absorbing at 57.47 (C-2), and the other chain carbons were assigned as $\delta$ 30.91 (C-3), 23.70 (C-4), 28.65 (C-5), 28.57 (C-6), 28.65 (C-7), 28.34 (C-8), 28.42 (C-9), 28.67 (C-10), 28.63 (C-11), 28.67 (C-12), 28.06 (C-13), 32.84 (C-14), 21.67 (C-15), 17.38 (C-16), 21.67 (C-17), 13.08 (C-18). Compound **5** was therefore identified as 2-ethylhexadecanoic acid.

Compound **6** was obtained as white crystal. The mass spectrum showed the presence of $[M + H]^+$ peak at *m/z* 175 corresponding to the molecular formula ($C_7H_{10}O_5$). Based on $^1$H-NMR and $^{13}$C-NMR spectral analyses, compound **6** was identified as shikimic acid.

Compound **7** showed a molecular peak at *m/z* 154 corresponding to the molecular formula ($C_7H_6O_4$). Based on $^1$H-NMR and $^{13}$C-NMR spectral analyses, compound **7** was identified as 3,4-dihydroxybenzoic acid.

Compound **8** was obtained as yellow amorphous powder. The mass spectrum of compound **8** showed a molecular ion peak at *m/z* 301.9, corresponding to the molecular formula ($C_{15}H_{10}O_7$). The IR spectrum showed absorption due to a carbonyl group at 1652 $cm^{-1}$ and a strong broad band at 3467 $cm^{-1}$ due to O-H stretching and a broad C-O vibration band at 1000 $cm^{-1}$. $^1$H-NMR (DMSO-$d_6$, 500 MHz) $\delta$: 6.17 (s, 1H, H-6), 6.404 (s, 1H, H-8), 7.54 (d, 1H, H-2′), 6.88 (d, 1H, H-3′), 7.66 (s, 1H, H-6′). $^{13}$C-NMR signals at 147.20 (C-2), 136.14 (C-3), 176.24 (C-4), 161.18 (C-5), 98.59 (C-6), 164.35 (C-7), 93.74 (C-8), 156.60 (C-9), 103.40 (C-10), 122.41 (C-1′), 115.42 (C-2′), 145.47 (C-3′), 148.10 (C-4′), 115.94 (C-5′), 120.40 (C-6′) demonstrated the characteristics of a

flavonol aglycone. Thus, compound **8** was identified as quercetin (Yao et al, 2013).

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